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FLUORESCENT CELL-COUNTING NEUTRALIZATION TEST FOR PSITTACOSIS

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ABSTRACT

A sensitive, precise, and specific serological procedure, the fluorescent cell-counting neutralization test, was developed to detect and to measure quantitatively psittacosis serum-neutralizing antibodies within 24 hours. The test is based on the reduction of fluorescent cells in McCoy cell monolayers resulting from the neutralization of infective virus particles by specific antiserum. Small but significant rises in neutralizing titers were measured in serum specimens from monkeys previously exposed to the virus and from humans with diagnoses of subclinical or established infections of psittacosis.

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I. INTRODUCTION

In comparison with the complement fixation and toxin neutralization tests and various modified forms of agglutination reactions, the serum neutralization test has not been generally employed in serological studies with members of the psittacosis group of microorganisms. An exception to this statement is the use of the neutralization reaction in studies on the immunological relationships among the psittacosis group made feasible by the production of serum of high neutralizing potency in immunized fowls.[1-4] An important factor predisposing to the limited usefulness of the neutralization test is the low levels or absence of neutralizing antibodies that have been encountered in serum derived from experimentally or naturally infected animals and humans.[3] In the majority of studies in which neutralization tests have been attempted, only qualitative estimates of the neutralizing capacity of serum have been obtained. The need for a sensitive and precise system to detect effectively and to measure quantitatively small but significant differences in neutralizing potency between sera is indicated.

In the course of developing a quantitative assay for psittacosis virus using the fluorescent cell-counting technique,[5] significant reductions of fluorescent cell counts were noted when psittacosis antiserum was mixed with the specific agent. A linear relationship was demonstrated between the quantity of virus neutralized and dilutions of antiserum. This finding, coupled with the high sensitivity, precision, and unusual rapidity of the assay (less than 24 hours), offered a system potentially capable of quantitatively determining serum-neutralizing antibodies against at least one member of the psittacosis group of agents. This report describes the standardization, application, and advantages of the fluorescent cell-counting neutralization test for psittacosis.

II. MATERIALS AND METHODS

A. VIRUS

The Borg strain of psittacosis virus was used throughout this study; its history has been recorded elsewhere.[6] A stock suspension of virus was prepared by infecting monolayers of McCoy cells with virus that had been passed once in this cell line. After incubation at 35 C for 48 hours, tissue culture fluids and cells were harvested, frozen and thawed once, clarified by low-speed centrifugation, dispensed into glass vials, and stored in an electric freezer at -60 C. The stock virus suspension assayed by the fluorescent cell-counting technique contained 1.1×10^7 cell-infecting units (CIU) per ml.

B. CELL LINE AND CULTIVATION

The established cell line, McCoy, derived from human synovial tissue[7] was used for assaying unneutralized virus particles. Nutrient medium for the cell line was mixture 199 containing 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and 50 and 75 µg streptomycin and kanamycin, respectively. Cells were maintained in mixture 199 and 5% calf serum. For virus assay, cells were cultivated on circular coverslips (15 mm diameter) inserted in flat-bottom glass vials (18 x 100 mm). One ml of cell suspension, containing 1×10^5 to 3×10^5 cells, was introduced onto coverslips that were then incubated at 35 C for 24 hours or until a complete cell monolayer was formed. Coverslip cultures were washed twice with 2 ml of maintenance medium prior to the addition of virus-serum mixtures.

C. ANTISERUM

Psittacosis hyperimmune serum was prepared by injecting roosters* intravenously with 1 ml of yolk sac suspension of virus containing $10^{7.0}$ egg LD_{50}. Three weeks later, surviving fowls were similarly injected and then bled 10 days after the last inoculation. An independent experimental study, in which *Macaca mulatta* monkeys were exposed to an aerosol of psittacosis (Borg) virus and bled periodically, constituted the source of paired monkey serum.[8] Human serum was obtained from individuals diagnosed as suspected subclinical or established infections of psittacosis on the basis of clinical and serological findings. Serum was heated at 56 C for 30 min before use in serological tests.

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* In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

D. NEUTRALIZATION TESTS

For carrying out the fluorescent cell-counting neutralization test, serial twofold dilutions of paired serum in phosphate-buffered saline (PBS) were mixed with an equal volume of a constant quantity of virus. The dilution of virus suspension employed contained a final concentration of 2×10^6 CIU in the inoculum. After mixtures were incubated at 35 C for 2 hours, 0.2 ml of each mixture was introduced onto 3 coverslip cultures of McCoy cells. Adsorption of unneutralized virus was carried out by centrifugation at 500 x g for 15 min, at 21 to 23 C. For this procedure, vials containing coverslip cultures were placed in slotted cups containing tube adapters, sealed with a screw-dome cover, and mounted on a 4-place, pin-type head. Centrifugation was performed in an International centrifuge, size 2, model V. Coverslip cultures were rinsed twice with maintenance medium following the adsorption period; 1 ml of the medium was then added to each vial. After incubation at 35 C for 20 to 22 hours coverslip cultures were rinsed twice with cold PBS, fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescence staining and cell counting or stored at -60 C for subsequent examination.

Fluorescent cell counts obtained from an examination of 50 microscopic fields for each coverslip culture were totalled, and the per cent reduction in counts by convalescent serum of the acute serum or appropriate control was calculated. From a plot of the logarithm of serum dilutions against the per cent reduction of counts straddling the 50% value, the 50% serum neutralizing endpoint was determined by interpolation.

In the mouse serum neutralization test, a similar protocol for the preparation of virus-serum mixtures was followed. The dilution of virus suspension, however, was adjusted to give a final concentration of CIU in the inoculum that was equivalent to that employed in the fluorescent cell-counting neutralization test. After incubation of virus-serum mixtures at 35 C for 2 hours, 0.03 ml of each mixture was then inoculated intracerebrally into 10 to 14 grams Swiss mice. Ten mice were inoculated for each virus-serum mixture. Animals were observed daily for 14 days and the survivors noted.

E. COMPLEMENT FIXATION TEST

The complement fixation test was carried out by the procedure described in the Lederle brochure on "Rickettsial and Viral Diagnostic Antigens."* Serum was titrated against two units of Lederle psittacosis antigen; titers were expressed as the highest dilution of serum exhibiting 100% inhibition of hemolysis.

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* Lederle Laboratories, Pearl River, New York.

F. IMMUNOFLUORESCENCE TECHNIQUES

The direct fluorescent antibody technique was employed to obtain immunofluorescence of infected cells. The preparation of psittacosis antiserum and conjugation with fluorescein isothiocyanate have been described previously.[6] Infected cell monolayers, fixed with cold acetone previously, were washed 3 times with PBS and stained with serum conjugate for 30 min. Coverslip cultures were rinsed then in three changes of PBS to remove excess conjugate and mounted in 10% glycerol in PBS. Coverslips were examined with an American Optical microscope equipped with a Fluorolume illuminator, model 645; Corning 5840 and Schott BG-13 exciter filters; and an E. K. 2A barrier filter. The number of microscopic fields contained in the area of a 15-mm coverslip was 1280 at 645 X magnification with this optical system. The number of CIU_5 per ml of virus suspension was calculated by the method described elsewhere.[5]

III. RESULTS

A. VIRUS ADSORPTION

Preliminary experiments revealed that prolonged contact between inoculum from virus-serum mixtures and cell monolayers during the period of virus adsorption resulted in an effect that was deleterious to the viability of cells 24 hours later. The effect occurred with mixtures containing high concentrations of serum when the adsorption period was carried out with cell monolayers maintained in a stationary position at 23 C or 35 C for 1 to 2 hours. Because attempts to estimate the quantity of unneutralized virus particles were precluded under these conditions, a rapid and efficient procedure for virus adsorption was imperative. An experiment was performed to determine the rate of virus adsorption onto cell monolayers during stationary incubation (35 C) and centrifugation (500 x g). To vials containing coverslip cultures, 0.5 ml of a 10^{-2} dilution of virus suspension was added. Vials were removed at designated intervals during the period of virus adsorption, and 0.25 ml of residual inoculum from each vial was introduced onto additional cell monolayers to measure the quantity of unadsorbed virus. Residual inocula derived from cell monolayers that had been previously centrifuged or held at stationary incubation were adsorbed at 500 x g, and 23 C for 1 hour, and 35 C for 2 hours, respectively. Following the designated periods of virus adsorption for both initial and residual inocula, all coverslip cultures were rinsed twice with maintenance medium, incubated, and fixed in the prescribed manner.

The per cent of virus adsorbed during each interval with each procedure is shown in Figure 1. Within 15 to 30 min, more than 98% of virus was adsorbed during centrifugation, whereas approximately 55% was adsorbed during stationary incubation for 2 hours. Since the efficiency and rapidity of virus adsorption onto cell monolayers attained by the use of centrifugal force was clearly superior to results with stationary incubation, centrifugation at 500 x g for 15 min at room temperature was employed as the procedure for adsorption of unneutralized virus in all subsequent tests. Cell monolayers were unaffected by high concentrations of serum in virus-serum mixtures using this procedure for the prescribed time.

B. THE 50% SERUM NEUTRALIZING ENDPOINT

Replicate twofold dilutions of hyperimmune rooster serum and an appropriate control were mixed with an equal quantity of virus suspension, incubated at 22 C for 1 hr, and introduced onto coverslip cultures in the manner described previously to determine the quantity of unneutralized virus particles. By plotting the logarithm of serum dilutions against

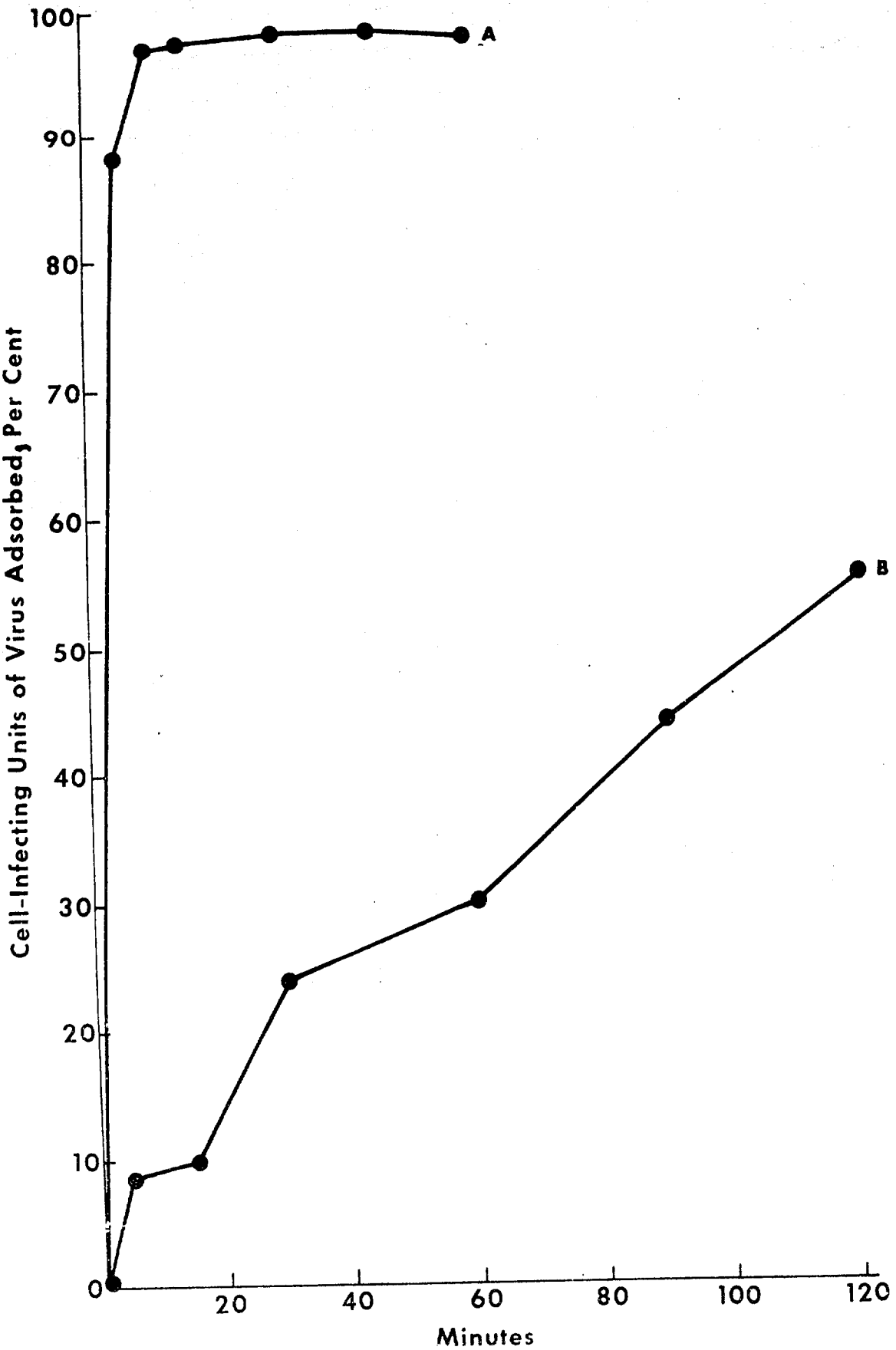

Figure 1. Adsorption of psittacosis virus onto coverslip cultures of McCoy cells by (A) centrifugation (500 x g, 23 C), and by (B) stationary incubation (35 C).

the per cent reduction of fluorescent cell counts for each dilution, a symmetrical curve is formed that is linear over a certain critical range on either side of the 50% reduction of cell counts (Figure 2). By interpolation, the 50% serum neutralizing endpoint can be determined accurately. The value of employing small dilution increments of serum in the proximity of the 50% fluorescent cell reduction point is obvious.

Nine determinations were made to estimate the precision of the fluorescent cell-counting neutralization test. The procedure of the test was similar to that described previously with the exception that virus-serum mixtures were incubated at 35 C for 2 hours prior to inoculation onto cell monolayers. The results in Table 1 reveal the unusually high precision of the test. The standard deviation, expressed as a percentage of the mean 50% serum neutralizing endpoint, was 3.3 with all endpoints ranging within one-half of this value.

C. INCUBATION OF VIRUS-SERUM MIXTURES

Because diverse opinions exist on whether incubation of virus-serum mixtures is necessary[9] varied conditions of incubation were tested to ascertain the effect on serum neutralizing endpoints. The results in Table 2 show that both time and temperature markedly influenced serum neutralizing titers. Neutralizing endpoints were approximately tenfold higher after incubation of mixtures at 35 C for 2 hours compared with 24 C for ½ hour. Prolonged incubation, especially at higher temperatures, reduced the fluorescent cell count of controls, which was indicative of virus inactivation. Since higher neutralizing titers were associated with reduced virus concentration of controls, it was necessary to determine whether these events were related. Three different quantities of virus were mixed with appropriate controls and a 1:128 dilution of antiserum; incubated at 23 C for 1 hour, and treated in the prescribed manner. Fluorescent cell counts from control mixtures containing varied quantities of virus were 1,427, 932, and 561; in the presence of antiserum, they were reduced 51.6, 58.0, and 52.1 per cent, respectively. The reduction of a constant percentage of virus, irrespective of the quantity present, by a given strength of antiserum reaffirms the operation of the "percentage law" described by Andrewes and Elford[10] and negates, in this instance, any relationship between reduced virus concentration of controls during incubation and serum neutralizing endpoints.

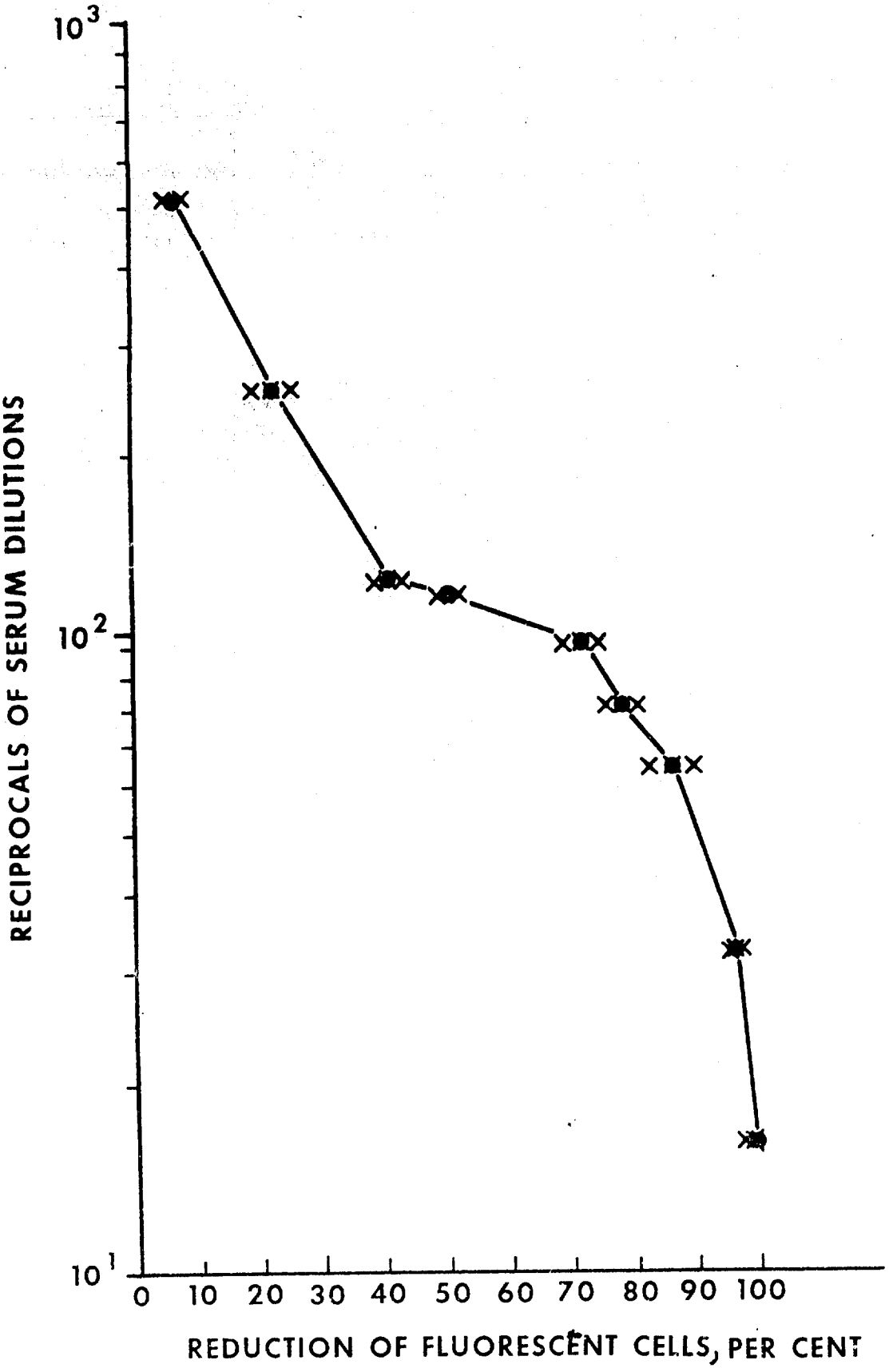

Figure 2. Curve relating per cent reduction in fluorescent cells by dilutions of hyperimmune serum in the fluorescent cell-counting neutralization test. Solid circles represent the mean of three determinations.

TABLE 1. PRECISION OF THE FLUORESCENT CELL-COUNTING NEUTRALIZATION TEST

Test	Serum[a] dilution 1:512 Count[b]	1:512 Per Cent Neutralizing[c]	1:1024 Count[b]	1:1024 Per Cent Neutralizing[c]	50% Serum Neutralizing Endpoint[d]
1	55	90.60	344	41.20	890
2	91	84.45	347	40.69	880
3	84	85.65	314	46.33	910
4	107	81.71	332	43.25	890
5	85	85.48	325	44.45	900
6	105	82.06	332	43.25	890
7	99	83.08	330	43.59	890
8	98	83.25	313	46.50	910
9	106	81.89	330	43.59	890
Arith. mean					896
Standard deviation					±30.4
Standard error of mean					±10.1

a. Test carried out with hyperimmune psittacosis rooster serum.
b. Total fluorescent cells in 50 microscopic fields.
c. Reduction of the number of fluorescent cells expressed as a percentage of control cell count of 585.
d. Reciprocal of serum dilution.

TABLE 2. EFFECT OF TIME AND TERMPERATURE ON SERUM NEUTRALIZING ENDPOINTS

Time, hour	Temperature, C	Fluor. cell count control	Serum[a] dilutions						50% Serum Neutralizing Endpoint[b]
			1:32	1:64	1:128	1:256	1:512	1:1024	
1/2	24	1041	122	303	619	662	674	928	105
1	24	932	6	68	402	515			180
2	24	669	22	38	64	325	401	583	280
2	35	518	8	2	7	4	72	157	>1024
2	4	873	152	223	213	331	492	539	400

a. Tests carried out with psittacosis hyperimmune rooster serum.
b. Reciprocal of serum dilution. Mean of 3 determinations.

D. COMPARATIVE SEROLOGICAL DETERMINATIONS

Complement fixation, fluorescent cell-counting neutralization, and mouse serum neutralization tests were employed to estimate the antibody content of paired serum from monkeys previously exposed to an aerosol of psittacosis virus. The results in Table 3 show the development of complement-fixing and serum neutralizing antibodies over a period of approximately 2 months. The former was first detected on the 19th day; the latter was noted on the 26th day after exposure of animals. Their order of appearance was in agreement with previous reported findings.[11] Complement-fixing titers were uniformly higher than serum neutralizing endpoints. In animals that had been exposed to virus approximately one or more months previously, however, an equivalent increase in geometric ratio of titers of both types of antibodies were noted. It is significant that serum-neutralizing antibodies were only detected by the fluorescent cell-counting neutralization test.

The results obtained from testing paired human serum by the same serological procedures are shown in Table 4. Both serum-neutralizing and complement-fixing antibodies were found in serum from patients in which the diagnosis of psittacosis was made previously on the basis of clinical findings and serological tests (patients 1 to 4). From suspected cases of psittacosis (patients 5 to 8) the highest increase in complement-fixing and serum-neutralizing titers, between early and later serum specimens, was only twofold. The possibility exists that higher serum neutralizing titers may have been demonstrated if the specific strains of psittacosis virus responsible for each infection had been known and available for use in the neutralization tests. In cases of psittacosis infection experienced 5 to 10 years before (patients 10 to 12), serum-neutralizing antibodies were detected in the absence of complement-fixing antibodies. Similar to the findings obtained with paired monkey serum, neutralizing antibodies were demonstrated only by the fluorescent cell-counting neutralization test.

No cross reactions were noted when the fluorescent cell-counting neutralization test was performed with monkey Q fever antiserum, rabbit variola, vaccinia, Coxsackie type A-9, Echo type 1, or poliovirus type 1 antiserum, or with human influenza type A, soluble antiserum.

TABLE 3. SEROLOGICAL DETERMINATIONS ON SERUM FROM MACACA RHESUS EXPOSED TO PSITTACOSIS VIRUS

Monkey	Serum	No. days after virus exposure	Serum Dilution Titers		
			CF test[a]	FCCNT[b]	Mouse SN test[c]
J 93	preexposure	0	<5	<2	
J 93	postexposure	0	<5	<2	
J 84	preexposure	0	<5	<2	
J 84	postexposure	7	<5	<2	
J 86	preexposure	0	<5	<2	<2
J 86	postexposure	17	20[d]	<2	<2
J 16	preexposure	0	<5	<2	<2
J 16	postexposure	19	640	<2	<2
J 22	preexposure	0	<5	<2	<2
J 22	postexposure	26	320	21	<2
G 13	preexposure	0	<5	<2	<2
G 13	postexposure	33	60	32	<2
J 90	preexposure	0	<5	<2	<2
J 90	postexposure	43	20	8	<2
L 33	preexposure	0	<5	<2	<2
L 33	postexposure	68	20	8	<2

a. Complement-fixation test.
b. Fluorescent cell-counting neutralization test.
c. Mouse serum neutralization test.
d. Numbers not preceded by < are reciprocals of serum dilution.

TABLE 4. SEROLOGICAL DETERMINATIONS ON HUMAN SERUM FROM PATIENTS WITH SUBCLINICAL OR ESTABLISHED PSITTACOSIS INFECTIONS

Comment	Patient	Date of serum sample	Serum Dilution Titers		
			CF test[a]	FCCNT[b]	Mouse SN test[c]
Clinical & serological diagnosis of psittacosis	1	Acute, DNR[d]	<4	<2	<2
		Convalescent, DNR	32[e]	8	<2
	2	Acute, DNR	<4	<2	<2
		Convalescent, DNR	128	2	<2
	3	Acute, DNR	<4	<2	<2
		Convalescent, DNR	32	2	<2
	4	Acute, DNR	NT[f]	<2	<2
		Convalescent, DNR	NT	2	<2
Suspected subclinical psitt. infection	5	7-1-63	<5	<2	NT
		12-19-63	10	2	NT
	6	12-18-61	<5	<2	<2
		12-16-63	10	4	<2
	7	12-5-61	<5	<2	<2
		12-17-63	10	4	<2
Technician working with psitt. virus	8	7-8-63	<5	<2	<2
		12-18-63	5	3	<2
Subclinical psitt. infection	9	4-15-64[g]	80	3	<2
Hosp. case of psitt.	10	2-10-64	<5	2	<2
Psittacosis 5-10 years previously	11	5-8-57	<5	2	<2
	12	5-18-64	<5	2	<2

a. Complement-fixation test.
b. Fluorescent cell-counting neutralization test.
c. Mouse serum neutralization test.
d. Date not recorded.
e. Numbers not preceded by < are reciprocals of serum dilution.
f. Not tested.
g. Acute serum sample not available.

IV. DISCUSSION

The wide devergency of methodology employed in neutralization tests has not fully resolved the general difficulty experienced in demonstrating low titers of serum-neutralizing antibodies that occur as a result of psittacosis infection.[3,4,12-16] Tests that were carried out employed different virus assays of varying sensitivity and accuracy, minimum or extended incubation periods for virus-serum mixtures, estimates of neutralization based on a variety of reactions and responses, and a protocol (constant serum-varying virus) for the preparation of virus-serum mixtures that required large changes in virus titer to reveal small differences in antibody concentration. In the fluorescent cell-counting neutralization test, the utilization of a protocol in which a constant quantity of virus is mixed with serial dilutions of serum provides a true measure of the amount of serum antibody present that can be expressed quantitatively as the 50% serum-neutralizing endpoint. In addition, by employing an assay for psittacosis virus of high accuracy (fluorescent cell-counting technique), an efficient procedure for adsorption of unneutralized virus onto cell monolayers (centrifugation), and an optimal incubation period for virus-serum mixtures (35 C for 2 hours); a sensitive, precise, and specific serological procedure is available that can be used to detect and measure small but significant differences in neutralizing antibody titers between two serum specimens. The ability to determine the neutralizing antibody content of serum in less than 24 hours is a singular advantage of the test.

The fluorescent cell-counting neutralization test may be applicable not only for diagnostic purposes but for retrospective epidemiological surveys, evaluation of the efficacy of vaccines, correlation between levels of neutralizing antibodies and development of resistance to infection, identification of virus strains, and studies to determine the immunological interrelationships among members of the psittacosis group of microorganisms.

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